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Characterization of Arsenic Compounds Formed by *Daphnia magna* and *Tetraselmis chuii* from Inorganic Arsenate

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Experiments to grow *Tetraselmis chuii* (a marine alga) and *Daphnia magna* in the presence of inorganic arsenate are described. The algae incorporate arsenic rather efficiently and form a lipid-soluble organic arsenic compound. *T. chuii* has been successfully mass cultured in a medium containing 10 ppm arsenic as arsenate. *Daphnia magna* was cultured in a medium containing ^{74}As-labeled H_3AsO_4 and 1 ppm Na_2HAsO_4 expressed as arsenic. The arsenic metabolites were extracted with a chloroform–methanol solution and isolated by using column and thin-layer chromatography. TLC analysis of the metabolites revealed the presence of a ^{74}As-containing product which migrated with phosphatidylethanolamine. This product was hydrolyzed with the phospholipases A, C, and D. The experimental results are not inconsistent with the presence of an arsenocholine moiety in the lipids. Arsenocholine, arsenobetaine, and acetylarsenocholine have been synthesized and will serve as reference substances in the chromatography experiments. The preparation of arsenocholine-containing lipids is in progress.

Introduction

Arsenic has been shown to be incorporated into both marine and fresh water organisms (*1*, *2*) in the form of water-soluble and lipid-soluble organic arsenic compounds (*2–4*). Although fish and higher members of the aquatic food chain seem to be capable of synthesizing organic arsenic compounds by themselves, organisms which are at the lower end of the food chain are, however, very likely the prime source of these compounds (*2*, *5*, *6*). The arsenic containing, lipid soluble compounds appear to be closely related to phospholipids (*3*), especially in their ability to produce hydrolysis products including arsenate (as compared to phosphate produced by phospholipids (*7*), to yield a positive ninhydrin reaction (*6*), and to migrate to R_f values which correspond to values characteristic of known phospholipids (*7*).

Because of the chemical similarities between arsenic and phosphorus, arsenic could perhaps replace phosphorus and form, for instance, an arsenolipid. Since arsenate esters are, in contrast to

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†Author to whom all inquiries should be addressed. To avoid the difficulties inherent in judging the relative importance of the contributions of each participant in this interdisciplinary effort usually expressed in the sequence of names appearing on this paper, the position of each name was determined in an unbiased manner with the aid of a table of random numbers.

phosphate esters, very easily hydrolyzed, arsenolipids are expected to be isolated only when all extractive operations are carried out under anhydrous conditions. It has been reported that hydrolysis of arsenic containing lipid samples extracted from cod and mackerel with chloroform–methanol (2: 1 v/v) yield two types of stable organic arsenic compounds: a water-soluble compound stable to 6.6*N* HCl (*8*) and a less polar compound which migrated in TLC experiments with the fatty acids (*9*). The identities of these lipid- and water-soluble organic arsenic compounds remain to be determined.

The investigations which are presently in progress at Texas A&M University and at the Agricultural Environmental Quality Institute, have as their goal the identification and characterization of the organic arsenic compounds formed by *Daphnia magna* and *Tetraselmis chuii* from inorganic arsenate. The two organisms were chosen because they are easy to grow, accumulate arsenic rather efficiently and are at the base of the food pyramid. Experiments with crayfish and lobsters were terminated because it was too difficult to maintain stable populations.

Our research group is presently engaged in three areas of activities, which in a synergistic manner should lead to the identification and isolation of the organic arsenic compound(s) formed by *Daphnia* and *Tetraselmis chuii:* large-scale culturing of *Tetraselmis* in the presence of arsenate to isolate gram quantities of the arsenic compound(s), small-scale experiments with *Daphnia,* and efforts to prepare arsenocholine, arsenobetaine, and arsenic-containing lipids.

Experiments with *Tetraselmis chuii*

The work of Lunde (*2, 6*) with marine algae made it possible to embark on a large-scale experiment to grow several hundred kilograms of algae in a salt water medium containing inorganic arsenate, extract the arsenic compound(s) with the lipids and separate the arsenic compound(s) from the lipids.

Twelve species of marine algae were first grown in test tubes in von Stosch and in Instant Ocean medium at arsenate levels (expressed as As) ranging from 0.5 to 50 ppm. *Tetraselmis chuii* was selected for large-scale experiments because of the organism's ruggedness and ability to efficiently take up arsenic. The growth behavior of *T. chuii* in the presence of arsenate ($Na_2HAsO_4 \cdot 7H_2O$) is shown in Figure 1. The organism grew well in media with arsenic concentrations as high as 50 ppm. The lag phase increased linearly with the arsenic concentration. Preliminary experiments employing media containing Na_2HAsO_4 spiked with gamma-active ^{74}As-H_3AsO_4 proved that *T. chuii* incorporates considerable amounts of arsenic into the cell (Fig. 2). If a similar arsenic accumulation occurs in the large scale growth experiments, 5000 liters of algae cultures will produce several grams of the arsenic compound(s).

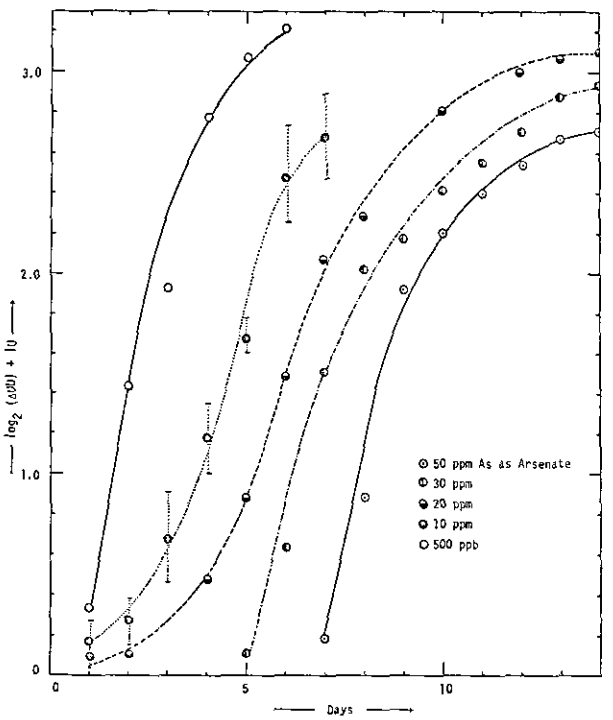


FIGURE 1. Growth of *Tetraselmis chuii* in arsenate-containing von Stosch medium: (○) 50 ppm As as arsenate; (○) 30 ppm As; (○) 20 ppm As; (●) 10 ppm As; (○) 500 ppb As.

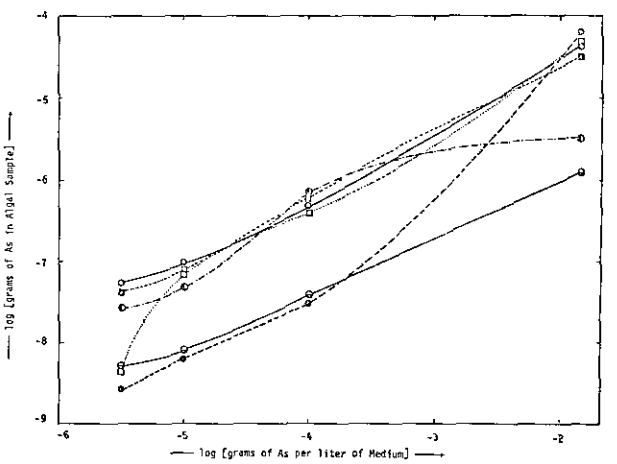


FIGURE 2. Amount of arsenic in algal samples (50 ml) harvested in their stationary phase as a function of arsenate concentration (as arsenic) in von Stosch medium: (○) Chlorella (1); (○) Syracasphaera; (■) Monochrysis; (○) Chlorella (2); (□) Tetraselmis; (●) Hemiselmis.

When a sample of *T. chuii* from a culture in the stationary phase in a low arsenic medium was transferred into a higher arsenic medium, the algae proceeded without much impairment through the three growth phases. By repeated transfer of algae into media of increasingly higher arsenic concentrations, organisms were obtained, which multiplied and thrived at 1000 ppm of arsenic. Transfer of these arsenic-tolerant algae into an arsenic-free medium caused the demise of the culture. Similarly, algae grown in arsenic-free medium did not survive when transferred into a 1000 ppm arsenic culture solution. Additional experiments in this area are underway.

Tetraselmis chuii was successfully mass cultured in four batches of 300 liters each in a medium containing 10 ppm of nonradioactive arsenic as arsenate. After 6 days, six liters of algae were separated by centrifugation, frozen, and stored for later processing. A room with appropriate environmental controls has been secured and four growing tanks, each holding 1000 liters, have been constructed. All necessary equipment for preparing artificial seawater and processing the algae has been installed. Large-scale culturing of algae will commence soon.

The methods of separating the lipids from the algae and isolating the arsenic compound(s) from the lipids are similar to the ones described for the Daphnia experiments. Analytical and preparative high-pressure chromatography will be used extensively in the final purification of the arsenic compounds. Preliminary results obtained with the arsenic compounds from algae suggest that they are similar to the one found in Daphnia.

Experiments with *Daphnia magna*

The Daphnia were cultured in an arsenic-free nutrient solution containing a thick growth of Euglena. Daphnia samples, estimated to weigh between 5 and 8 g were then removed and placed into another culture medium containing NaH_2AsO_4 (1 ppm As) and ^{74}As–H_3AsO_4. After 3 days the Daphnia were harvested, washed with distilled water, air dried, and then treated with cold trichloroacetic acid–water (5:95, v/v) to remove low molecular mass compounds (*10*). The separated solids were washed with distilled water and air dried. The air-dried sample was extracted (*11*) with $CHCl_3/CH_3OH$ (2:1, v/v). The chloroform layer, which contains the lipids was analyzed by column chromatography (silica gel) with $CHCl_3/CH_3OH/H_2O/CH_3COOH$ (65:25:4:6v/v) as eluent and by dry column chromatography (*12*). Figure 3 summarizes these operations in a flow-chart.

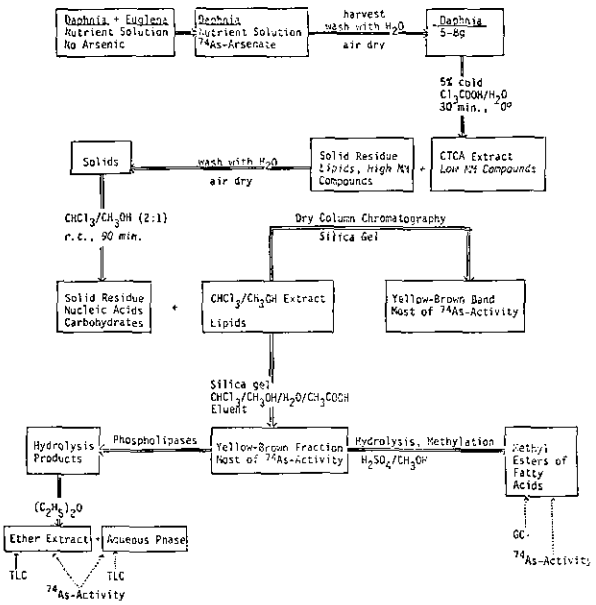


FIGURE 3. Flow chart for the isolation of organic arsenic compounds from *Daphnia magna*.

The lipid-containing chloroform layer accounted for approximately half of the ^{74}As activity present in the Daphnia. A dry column chromatographic separation of this extract produced a yellow-brown band with R_f 0.58 which contained most of the ^{74}As activity. An appreciable amount of radioactivity remained at the top of the column.

Another sample of the chloroform layer was loaded on a silica gel column and eluted with the $CHCl_3/CH_3OH/H_2O/CH_3COOH$ mixture. The yellow-brown eluate was further separated by TLC with the above solvent mixture. The chromatogram after spraying with ninhydrin solution revealed two violet spots, one at R_f 0.57 (62%) and another of less intensity of R_f 0.29 (15%). With dichlorofluorescein under ultraviolet light, four more spots at R_f 0.10 (5%), 0.34 (7%), 0.67 (8%), and 0.79 (13%) were detected. The percentages in parentheses represent the fraction of the total ^{74}As activity on the TLC plate found with the particular spots. The ninhydrin-positive spot at R_f 0.57 contains most of the activity and has the same R_f value as the yellow-brown band on the dry column.

Various phospholipids and a sample of the yellow-brown eluate were simultaneously chromatographed two-dimensionally, first with $CHCl_3/CH_3OH/NH_3$ and then with the $CHCl_3/CH_3OH/H_2O/CH_3COOH$ system. The ninhydrin-positive spots with R_f 0.57 and R_f 0.29 migrated in this experiment to the same R_f values as phosphatidylethanolamine and lysophosphatidylethanolamine, respectively.

When the TLC analysis of the yellow-brown fraction was performed immediately after elution only the spot with R_f 0.57 was detected. A thin layer chromatogram of a 3-day-old sample of the eluate even when stored under nitrogen at 0°C showed all six spots described above. Storage of the sample under nitrogen at −12°C prevented the formation of the compounds responsible for the additional five spots.

The yellow-brown fraction was subjected to enzymatic hydrolysis by phospholipase A, C, and D (*13–15*). The hydrolysis mixture was extracted with diethyl ether. Both, the organic and the aqueous phase were characterized by TLC and counted for ^{74}As-activity.

The following ^{74}As-activity distribution between the aqueous and the ether phase was found [phospholipase (PL), % activity in H_2O, % activity in ether]: PL A 98%, 2%; PL C, 3%, 97%; PL D 43%, 57%. Upon total hydrolysis and methylation with CH_3OH/H_2SO_4, 7% of the activity was found in the ether phase and 93% in the aqueous layer.

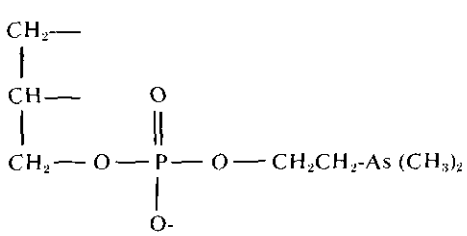
When the lipids were cleaved by PLA, the reaction mixture concentrated and a sample chromatographed, a ninhydrin-positive, ^{74}As-active TLC spot was observed. The ether extract contained palmitic acid but little radioactivity.

Hydrolysis by PL C and extraction produced an ether layer containing 97% of the activity. TLC analysis of the ether layer produced a ninhydrin-negative, ^{74}As-active spot which tailed from the origin to R_f 0.10.

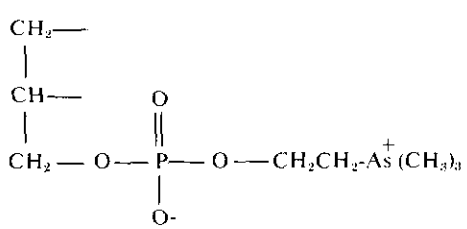
The arsenic-containing materials in the PL D hydrolysis mixture partitioned about equally between the ether and the water layer. The TLC of the ether layer gave a ninhydrin-negative, ^{74}As-active spot at R_f 0.39, a ninhydrin-positive, but inactive spot at R_f 0.67, and a ninhydrin-negative, inactive spot with $R_f \sim$ 1.0. The material in the aqueous layer, which was ninhydrin-positive and radioactive, did not migrate.

A sample of the aqueous layer obtained upon complete hydrolysis of the yellow-brown eluate spotted on TLC and developed with $CHCl_3/CH_3OH/NH_3$ similarly did not migrate. The spot was radioactive and ninhydrin-positive. Based on these very preliminary results, suggestions as to the nature of the organic arsenic compounds formed by Daphnia can be made. The further experiments, which are now being carried out, will show whether or not these suggestions have any merit.

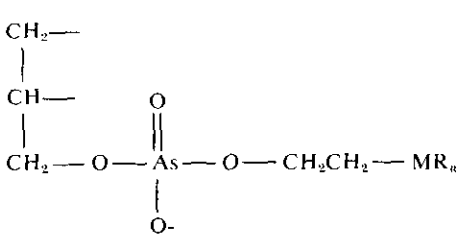
Structures I–VI suggest how arsenic could be incorporated into lipids. It is of course possible that arsenic does not appear as part of a lipid molecule but has been transformed into an arsenic compound, whose properties allow it to be separated together with the lipids. At the moment, it seems to be expedient to let the arsenic atom become part of a lipid molecule and take the place of the nitrogen atom in the choline group bonded to glycerine via a phosphate group.



I



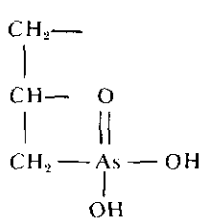
II



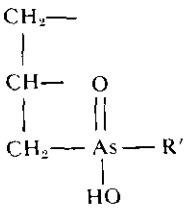
M = N,As

n = 2,3

III

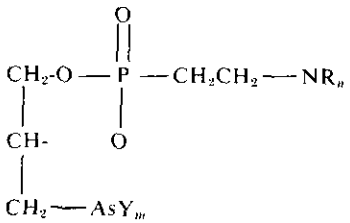


IV



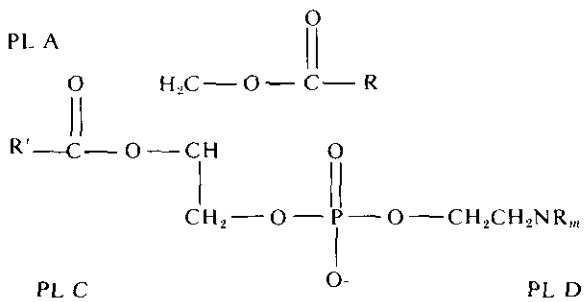
V

R′ = alkyl group



VI

Y = organic group, OH
m = 2, 3



VII

Considering the sites of attack of the phospholipases as shown in VII, an attempt can be made to explain the experimental results on the basis of the structures and likely properties of the arsenic-containing lipids I–VI. Let us assume then, that the arsenic is present in the lipid molecule as a replacement for nitrogen in choline but not in ethanolamine. The AsH_2 group is extremely oxygen sensitive and toxic. Organisms probably prefer methylated arsenic groups. Such a lipid could have formula II or perhaps I.

PL A hydrolysis will remove the β-carboxylic acid in the choline-containing lipid and perhaps in the arsenocholine-containing lipid. Since the N- and As-lipids seem to have similar TLC properties, a ^{74}As-active, ninhydrin-positive (from lysophosphatidylethanolamine) spot would be expected. The experiment produced such a spot with a R_f value of 0.29.

PL C cleaves the phosphoethanolamine group. It is conceivable that a compound with arsenic in the place of nitrogen is for steric reasons not able to serve as a substrate for the enzyme. The arsenic-lipid would not be hydrolyzed. It would move into the ether layer and not give a ninhydrin reaction as observed.

PL D gave a hydrolysis mixture, half of whose activity partitioned into the ether layer. The phospholipid will lose its ethanolamine group, which will be soluble in the aqueous phase. Perhaps the enzyme does interact with, for instance compound II, but at a rate slower than that characteristic for the nitrogen derivative. The arsenocholine would go into the aqueous phase, the intact arsenic lipid would stay in the ether phase as observed. Add total hydrolysis in pursuit of our third activity in this area.

All these experimental results are not incompatible with the presence of an arsenocholine group in a lipid of structure II. The data available at this time allow, of course, no definite statement at all about the mode of occurrence of arsenic in lipids. Our preliminary, tentative and intriguing "arsenocholine" hypothesis does, however, provide some guidance in our search for the elusive organic arsenic compounds formed by algae and Daphnia.

We have recently prepared arsenocholine and arsenobetaine and are working on the synthesis of arsenic lipids of the type I, II, IV, and V. Enzymatic hydrolysis reactions will be carried out with these compounds. They will also be used as reference derivatives in the chromatographic investigations.

The frequently mentioned possibility of arsenate replacing phosphate in biologically important molecules leads to the question: could lipids be formed in which arsenate replaced phosphate as in compound III? Such a derivative could be stored only under anhydrous conditions and would certainly not survive the extraction procedures which

are not at all anhydrous. Arsenate esters are extremely easily hydrolyzed. Several of the aqueous hydrolysis solutions were chromatographed simultaneously with arsenate and the chromatogram sprayed with silver nitrate. Arsenate was not detected in the hydrolysis mixture. No radioactivity was found on the chromatogram of the hydrolyzate corresponding to the arsenate region. The replacement of phosphate by arsenate in lipids is, therefore, considered to be unlikely.

Since phosphonolipids do occur in nature, arsonolipids could exist as well. These compounds IV and VI and the arsinolipid V have all an arsenic-carbon bond. As long as there is not a hydroxyl group in β-position to the arsenic atom the derivatives should be stable. Hydroxyethyl arsenic compounds have a tendency toward arsenic–carbon bond cleavage under relatively mild conditions. If an arsono- or arsinolipid (IV, V) had been present, PL D would have had no effect on the molecule.

The situation becomes, of course, more complex when a compound of type VI is considered, especially if arsenic also replaces the nitrogen atom in this lipid molecule.

We believe that the nature of the lipid-soluble organic arsenic compounds will be known soon. Whether arsenocholine is one of these compounds remains to be seen.

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